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Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure

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Objective: To reexamine whether low sperm HspA2 ratios (formerly *sperm CK-M ratio*) are predictive for failure to cause pregnancies by in vitro fertilization (IVF) and to explore whether there are other male or female factors that may be predictive for IVF pregnancy outcome.

Design: Retrospective, cohort study.

Setting: University-based IVF program.

Patient(s): In 119 IVF cycles, three patient groups were studied: 25 men had a <10% sperm HspA2 ratio and a lack of pregnancies (HS <10% group), 50 men had a >10% sperm HspA2 ratio and no pregnancies (HS >10%NP group), and another 44 couples had a >10% sperm HspA2 ratio but did achieve pregnancies (HS >10%P group).

Intervention(s): Sperm HspA2 ratio determinations within 1 year of the IVF cycles and analysis of male and female IVF cycle parameters.

Main Outcome Measure(s): Sperm HspA2 ratio determinations within 1 year of the IVF cycles and analysis of male and female IVF cycle parameters.

Result(s): In the three groups, male and female ages, number and maturation level of oocytes, and morphology of embryos were similar. In the HS < 10% group, mean sperm concentration and motility were close to normal, the fertilization and cleavage rates were lower, and cycles without any oocyte fertilization were higher. These parameters were similar in the two HS > 10% groups. The receiver operating characteristic curve in men with sperm HspA2 ratios of <17% (diminished and borderline sperm maturity) provided a cutoff value of 10.84% HspA2 ratio with a 100% positive predictive value for failure to achieve pregnancy, whether the men were oligospermic or normospermic.

Conclusion(s): HspA2 ratios of <10% in the diminished sperm maturity range predict the failure to cause pregnancies by IVF. Thus, IVF should be bypassed in favor of ICSI. The occurrence of pregnancy with ICSI depends on the maturity of sperm selected, and it may not be as likely as in other indications for ICSI. (Fertil Steril® 2002;77:910–8. ©2002 by American Society for Reproductive Medicine.)

Key Words: Sperm function, HspA2 chaperone, IVF pregnancy, sperm biochemical markers, paternal contribution

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The lack of reliable methods to assess sperm-fertilizing potential has been a long-standing problem for infertile patients and their physicians. The conventional semen parameters, including sperm concentrations, motility, velocity, and morphology, are often of limited utility. It has become increasingly obvious that there is a need to identify cellular markers of sperm cellular maturity that would predict fertilizing potential independently from the semen analysis criteria. Measurements of sperm creatine-*N*-phosphotransferase or creatine kinase (CK) have revealed significantly higher sperm CK activities in oligozoospermic men with diminished fertility than in fertile normozoospermic men (1). We have addressed the reasons underlying the sperm CK activity differences with CK immunocytochemistry in individual spermatozoa (2). The immunostaining patterns indicated that high sperm CK activity was a

direct consequence of increased CK concentrations in the immature spermatozoon. The combination of increased cytoplasmic CK concentrations and diminished sperm function suggested to us that we had identified a sperm developmental defect in the last phase of spermiogenesis, when the cytoplasm (unnecessary for the mature sperm) is normally extruded and left in the adluminal area as residual bodies (3).

The idea of incomplete sperm cellular maturation led us to analyze the constituent sperm proteins and to make an important discovery. In addition to the CK marker, we found a second developmentally regulated protein that at the time we thought to be a unique sperm-specific CK-M isoform but it has subsequently been identified as the 70-kDa testis-expressed chaperone protein, HspA2. The HspA2 levels were lower in sperm with high CK activity (4, 5). This evidence has further supported the concepts that [1] the increases in CK and cytoplasmic protein content are markers of unextruded cytoplasm and arrested development and that [2] diminished HspA2 expression during spermiogenesis is another indicator of incomplete sperm maturation. In line with these ideas, there was a close correlation between the decline in sperm CK activity and a developmentally regulated increase of HspA2 in three large independent studies ($r = -0.69$, $r = -0.70$, $r = -0.72$; $P<.001$ in all three; N = 159, 115, and 145) (4, 6, 7). It is of further interest that in the rodent model, selective destruction of the gene for the 70-kDa chaperone protein, homologous to the human HspA2, caused diminished germ cell maturation and sperm production (7).

The degree of sperm maturity in a sample is best detected by the HspA2 ratio, which is based on measurements of the adenosine triphosphate (ATP) associated with HspA2 and CK. For the determination of the HspA2 ratio, the HspA2 (contains bound ATP) and CK (generates ATP from adenosine diphosphate and creatine phosphate in sperm) are separated by agarose gel electrophoresis and overlain with a fluorescent ATP substrate. The fluorescent bands are scanned, and the HspA2 ratio is determined according to the integrated proportion of fluorescence under the HspA2 and CK peaks. We express this ratio as follows: %HspA2/(HspA2 + CK-B).

We have previously conducted two clinical studies examining the utility of sperm CK-B activity and HspA2 ratios in the assessment of male fertility or infertility. In couples treated with intrauterine insemination, the sperm CK activities were lower ($P<.001$) in oligozoospermic male partners who achieved pregnancy compared with those who failed to cause pregnancy. A logistic regression analysis based on 160 samples showed a significant negative correlation between sperm CK activity and the occurrence of pregnancy, whereas the sperm concentrations in the couples provided no predictive power (9). Others have also confirmed the value of sperm CK measurements in the evaluation of sperm function (10–12).

The predictive value of HspA2 ratios was tested in a blinded study of 84 couples undergoing IVF at the Yale Center for Reproductive Medicine and at Norfolk in 1990/1991 (6). We classified the 84 men based only on their HspA2 ratios (then known as *CK-M ratios*), without any information on semen parameters or reproductive history, into groups labeled *low likelihood* (<10% HspA2, N = 22 men) and *high likelihood* (>10% HspA2, N = 62 men) for fertility. All pregnancies occurred in the high-likelihood group. No pregnancy occurred in the low-likelihood <10% HspA2 group. Among the couples in the high-likelihood group, if at least one oocyte was fertilized, suggesting that the wife did not have an oocyte deficiency, the pregnancy rate was very high (30.4% per cycle in 1990 and 1991).

The HspA2 ratio offered another important use as well: 9 of the 22 low-likelihood men were normozoospermic but had diminished sperm fertility (6). Thus, the HspA2 ratio provided a diagnostic tool for the detection of men who had diminished sperm fertility despite having normal semen parameters.

Men undergoing treatment at the Yale Center for Reproductive Medicine are routinely evaluated for sperm CK and HspA2 ratio parameters. In light of the changes in the IVF treatment of male factor infertility, we have reexamined the role of HspA2 ratios in the assessment of male fertility and the potential role of the HspA2 ratios in the clinical decision of whether a couple should be treated with IVF or ICSI. The objectives of this study were to [1] determine whether a <10% sperm HspA2 ratio in male partners of infertile couples (HS <10% group, N = 25), irrespective of otherwise adequate motile sperm concentrations, is predictive for the failure to achieve pregnancy in conventional IVF; [2] confirm that there are men with diminished sperm maturity and fertilization potential, despite normospermic semen parameters; and [3] determine whether, in addition to couples with male partners with >10%HspA2 ratios who did not (HS > 10%NP group) or did (HS > 10%P group) achieve pregnancies, respectively, there are any other male, female, or IVF parameters that are predictive for failure or success in achieving pregnancies.

MATERIALS AND METHODS

Patient Selection

This study was performed retrospectively by analyzing couples undergoing conventional IVF but not ICSI cycles at the Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Yale School of Medicine between January 1993 and December 1999. In the ensuing period, we have performed 1370 diagnostic HspA2 studies, and 104 of these men had HspA2 ratios of <10%. Among these couples, 66 underwent treatment: 25 with conventional IVF and 41 with ICSI. For the present study, we selected the couples with a sperm HspA2 ratio of <10% and regular IVF cycles

TABLE 1

Semen parameters, sperm CK activity, and HspA2 ratio in the three groups.

Parameter	HS <10% (n = 25)	HS >10%NP (n = 50)	HS >10%P (n = 44)
Normospermic samples, n (%)	13 (52%)	42 (84%)	38 (86%)
Semen volume, mL (range)	2 ± 0.2[a] (1.61–2.49)	2.5 ± 0.1 (2.26–2.78)	3 ± 0.2 (2.63–3.35)
Sperm concentration ($\times 10^6$/mL)	31.3 ± 6.6[b] (17.73–44.79)	77.1 ± 6.9 (63.37–90.80)	83.2 ± 9.6 (63.84–102.52)
Sperm motility (%)	42 ± 3.4[b] (34.91–49.11)	53.4 ± 2.1 (47.41–60.41)	56 ± 2.3 (50.41–64.57)
Total motile sperm	35 ± 10.7[b] (12.54–56.67)	98.6 ± 9.3 (79.88–117.28)	124 ± 13.6 (96.52–151.44)
World Health Organization morphology Abnormal (%)	33.3 ± 2.2 (28.72–37.84)	34.9 ± 0.5 (33.83–35.93)	33.9 ± 0.9 (32.04–35.73)
HspA2 ratio (%)	7.6 ± 0.5[b] (6.70–8.58)	45.2 ± 2.8 (39.61–50.83)	39.2 ± 2.9 (33.41–45.02)
CK activity (IU/10^8 sperm)	0.9 ± 0.1[b] (5.93–11.31)	0.14 ± 0.02 (1.04–1.75)	0.17 ± 0.02 (1.22–2.17)

Note. Unless otherwise noted, values in parentheses are 95% confidence intervals. Yale Sperm Physiology Laboratory normal values: Semen volume, >1.5 mL; sperm concentration, >20 million/mL; sperm motility, >40%; total motile sperm, >12 million; WHO sperm morphology, <40% abnormal sperm.

[a] $P < .05$; HS <10% vs. HS >10%NP and HS >10%P. No differences between HS >10%AP and HS >10%P.

[b] $P < .001$; HS <10% vs. HS >10%NP and HS >10%P. No differences between HS >10%NP and HS >10%P.

Ergur. Sperm HspA2 ratio and IVR pregnancy outcome. Fertil Steril 2002.

within a 1-year duration before or after the HspA2 ratio determination (HS < 10% group).

As control, we selected the couples undergoing IVF treatment within 1 month of those in the above HS <10% group; the men in these control couples had sperm HspA2 ratios in the >10% range. This control group was further divided into two groups according to their failure (HS >10%NP) or success (HS >10%P) in achieving an IVF pregnancy. Other than the 1-year time window, further exclusion criteria included IVF cycles with <2 oocytes retrieved, female partners with Day 3 FSH levels of >15 mIU/mL, use of donor oocyte and sperm, as well as female partners aged >45 years. Each couple had a standard infertility workup, including semen analysis and sperm HspA2 determinations.

The semen analyses were carried out after 2–3 days of abstinence. After a 30- to 40-minute liquefaction period, followed by thorough mixing of the semen, an aliquot of 3–5 μL of semen was placed in a Makler chamber. The operator evaluated four fields in two different drops (more fields in low–sperm concentration samples) to determine sperm concentration and motility using the IVOS CASA instruments (Hamilton-Thorne, Beverly, MA). The semen analysis results are reported in Table 1.

The study was carried out in a blinded fashion: all IVF, pregnancy outcome, and female-partner–related data were analyzed before the male partner's HspA2 ratios were disclosed. All sperm biochemical studies were approved by the Yale School of Medicine's Human Investigation Committee.

Sperm CK Activity and HspA2 Ratio Determinations

These assays were performed by standard procedures, as described previously (13). Aliquots of semen were washed with 10–15 volumes of ice-cold 0.15 M NaCl and 30 mM imidazole (pH 7.0) at 5000 × *g* to remove seminal fluid, and the sperm pellets were disrupted by vortex processing in 0.1% Triton, 30 mM of imidazole (pH 7.0), 10% glycerol, and 5 mM of 1,4 dithiothreitol. The homogenate was clarified by centrifugation at 5000 × *g*, and aliquots of the sperm extract were subjected to CK activity determinations by a spectrophotometric CK kit (Sigma Co., St. Louis, MO).

The two sperm proteins, CK and HspA2, were separated by electrophoresis on agarose gels (Helena Laboratories, Beaumont, TX), and the ATP associated with the proteins was detected by overlaying and incubating the gel with a fluorescent ATP substrate for 20 minutes at 37°C. The fluorescent bands were scanned and integrated under long-wave ultraviolet light with a Helena Scanning Fluorometer. The HspA2 ratios were determined according to the integrated areas representing the proportion of fluorescence under each peak. The intra-assay coefficient of variation for the HspA2 ratio assays is approximately 8%.

Cut-Off Value for the HspA2 Ratios in Men With Diminished Sperm Maturity

We have previously established that the upper limit of normal CK activity is 0.25 CK IU/10^8 sperm (based on the mean + 2 SD of the sperm CK activity in a population of 180 men) (1). In three studies on the CK and HspA2 parameters of >400 men, there were significant inverse correlations between CK activities and the HspA2 ratios ($r = -0.69$, -0.70, and -0.72; $P<.001$ in each), which reflect the close relationship between the spermiogenetic events of cytoplasmic extrusion and the expression of the HspA2 isoform (4, 6, 7). In the present study, this correlation was $r = -0.82$; $P<.001$; N = 119 (discussed later in this article). Considering that the 0.25 CK IU/10^8 sperm activity is the upper limit of the normal range for CK, we located the HspA2 ratio value that correlated with the 0.25 CK IU/10^8

sperm activity, which was approximately 10%. The 10% HspA2 ratio was therefore used as a cutoff value of the diminished maturity range in both the 1992 study and the present one.

Characteristics of the Patient Population

A total of 119 IVF couples, who also provided semen for sperm HspA2 determination, were studied in terms of female and male characteristics, semen assessment, sperm CK activity, and HspA2 ratios. All couples underwent conventional IVF with day 3 transfers, and couples treated with ICSI cycles were not considered.

Regarding the indications for IVF, we have distinguished couples who have [1] male factor infertility: oligozoospermia of <20 million sperm/mL or asthenozoospermia of <40% sperm motility; [2] ovulatory dysfunction: female partners with unresponsive basal body temperature, absence of LH surge, low luteal progesterone, or repeated months with irregular menses; [3] immunologic infertility: couples with anti-sperm antibodies in the male or female; [4] tubal disease; [5] endometriosis; and finally, [6] poor response: female partners with elevated day 3 FSH and E_2, indicating poor ovarian reserve. This group also included women who showed low levels of serum E_2 and follicular development after administration of adequate gonadotropin stimulation.

IVF-ET Procedures

All patients underwent pituitary down-regulation with GnRH agonists such as leuprolide acetate (Lupron; TAP Pharmaceuticals, Chicago, IL) in the midluteal phase of the previous cycle according to the long-stimulation protocol. Once pituitary suppression was achieved, controlled ovarian hyperstimulation was initiated with either human menopausal gonadotropin (Pergonal; Serono, Norwell, MA) or with purified or recombinant FSH (Gonal-F; Serono) on day 3 of the following cycle and continued until the day of hCG (Profasi; Serono) administration. When at least three follicles were ≥18 mm in diameter, hCG was administered (10,000 IU IM), and oocytes were retrieved 34–36 hours later.

Insemination and sperm preparation using human tubal fluid medium (Irvine Scientific, Irvine, CA) was routinely performed between 11 A.M. and 2 P.M. The following morning (day 1), fertilization was assessed by examining the oocytes for the presence of two pronuclei and polar bodies. Further analysis of embryo cleavage was performed 24 and 48 hours later. The embryo transfer (average of 3 embryos, but without a maximum limitation) was done on day 3. After retrieval, we evaluated the oocyte–cumulus complex by using a grade scale of 1–3, which corresponds to states as follows: 1 = immature, 2 = intermediate, 3 = fully mature and expanded. Finally, embryo maturity was assessed by grading 1–5, in which 1 corresponds to the best and 5 to the worst embryo morphology.

Luteal support with progesterone (100 mg), intramuscularly or by vaginal gel (Crinone; Wyeth-Ayerst, Philadelphia, PA), was initiated on the day of the transfer. Serum β-hCG levels were measured 2 weeks later, and on positive findings (>100 IU/mL), the β-hCG levels were followed by weekly assays. An ultrasound examination was conducted on the β-hCG titers reaching the >2000 IU/mL level. Patients in whom the fetus displayed a heartbeat by ultrasound examination were considered to have achieved a clinical pregnancy.

Statistical Analysis

All the values were expressed as mean ± SEM. To determine the differences among the groups and to make the comparisons, we used Student's *t*-test, χ^2, and one-way ANOVA analyses, and the post hoc tests of Tukey and Scheffe, using the computer-based statistical program SigmaStat. The correlations were done by using the Pearson product moment correlation test. The receiver operating characteristic (ROC) curve was generated by the GraphROC for Windows program (Kairisto and Poola, Turku, Finland). Statistical significance was set at $P<.05$.

RESULTS

Characterization of the Study Population

The main characteristics of the three groups (HS < 10%, HS > 10%NP, and HS > 10%P), divided according to the sperm HspA2 ratios and pregnancy success, are summarized in Tables 1 and 2. As the data indicate, no statistical difference was observed among the couples regarding female and male ages or the IVF indications, with the exception of a higher proportion of men with male factor infertility in the HS < 10% group. However, even in the HS < 10% group, the mean sperm concentration parameters were in the normozoospermic range, and the sperm motility was >40% (Table 2). Indeed, among the 25 men of the diminished-maturity HS < 10% group, 13 (52%) were normozoospermic.

In line with the cutoff value of <10% HspA2 ratio, we found significant differences between HS <10% and the two HS >10% groups in HspA2 ratios. However, there were no differences between the last two groups. Similarly, the other biochemical marker, CK activity, which reflects the excess unextruded cytoplasm in sperm with diminished maturity, showed differences between the HS <10% group and the two HS >10% groups but no differences between the HS >10%NP and HS >10%P groups. As in the previous HspA2 and CK studies, there was a close and highly significant correlation ($r = -0.82$, $P<.001$) between CK activity and HspA2 ratio, further strengthening the concept that the synthesis of HspA2 and the extrusion of the cytoplasm are related maturational events during the last phase of spermiogenesis.

In addition to establishing the differences in mean sperm concentrations between the HS <10% and the two HS >10%

TABLE 2

Characteristics of the patient population (mean ± SEM).

Characteristic	HS <10% (n = 25)	HS >10%NP (n = 50)	HS >10%P (n = 44)
Female age, y	33.8 ± 0.8	34.7 ± 0.6	33.6 ± 0.6
Male age, y (IVF indications)	36.5 ± 1.2	36.3 ± 0.6	37.1 ± 0.7
Male factor, n (%)	6 (24)	3 (6)	4 (9)
Ovulatory dysfunction, n (%)	2 (8)	1 (2)	4 (9)
Tubal disease, n (%)	10 (40)	24 (48)	25 (57)
Immunologic infertility, n (%)	—	1 (2)	1 (2)
Endometriosis, n (%)	—	10 (20)	6 (14)
Poor responder, n (%)	3 (12)	1 (2)	—
Uterine factor, n (%)	—	1 (2)	—
Unexplained Infertility, n (%)	4 (16)	9 (18)	4 (9)

Note: No difference was observed between the groups within male or female groups.

Ergur. Sperm HspA2 ratio and IVF pregnancy outcome. Fertil Steril 2002.

groups, we also considered the distribution of sperm concentrations in the samples (Table 3). There was one sample each in the HS <10% group and, notably, in the HS >10%P groups in the range of <5 million sperm/mL, and there was a higher proportion of oligozoospermic samples in the 5–10 million sperm/mL range in the HS <10% group. In the HS <10% group, all but two men (who respectively had 0.9 and 1.8 million total motile sperm) had total motile sperm concentrations of >3 million, a level that provides, considering the oocyte insemination step, sufficient chance for IVF success. Thirteen samples ranged from 3 million to 18 million sperm, and the other 10 men had total motile sperm concentration of >18 million. In the 94 men of the two >10% HspA2 ratio groups, who had a 47% pregnancy rate overall, 96% or more of the samples were in the >18 million sperm range. Again, similar to the case with other sperm parameters, there was a difference in the HS <10% group compared with the HS >10%NP and HS >10%P groups, but 13 men (52%) in the HS <10% group were normozoospermic, with diminished sperm maturity <10% HspA2 ratio.

None of the other sperm data were different between the HS >10%NP and HS >10%P groups, indicating that the various semen parameters would not be predictive for IVF success or failure, if the sperm maturity of the male partner is adequate. The distribution of pregnancies among the 44 couples further support the importance of the proportion of the mature sperm in the samples. Five pregnancies (11%) occurred within the 10%–17% HspA2 ratio range, and 27 (62%) were by men in the >30% sperm HspA2 ratio range (Fig. 1).

TABLE 3

Sperm concentrations and the total motile sperm in the three groups.

Sperm parameter	HS <10% (N = 25)	HS >10%NP (N = 50)	HS >10%P (N = 44)
Sperm concentration (sperm × 10^6/mL)			
< 5	1	0	1
6–10	6	1	0
11–15	2	1	0
16–20	3	0	0
>20	13 (52%)	48 (96%)	43 (98%)
Total motile sperm (million)			
<3	2 (8%)	0	0
3–18	13 (52%)	2 (4%)	3 (7%)
>18	10 (40%)	48 (96%)	41 (93%)

Note: Data in parentheses are percentages.

Ergur. Sperm HspA2 ratio and IVF pregnancy outcome. Fertil Steril 2002.

FIGURE 1

Occurrence of pregnancies vs. sperm HspA2 ratio ranges.

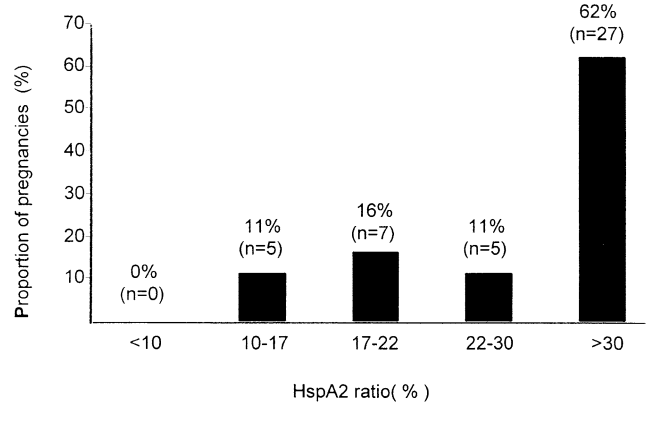

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TABLE 4

IVF cycle parameters of the groups.

Parameter	HS <10% (n = 25)	HS >10%NP (n = 50)	HS >10%P (n = 44)
No. of retrieved oocytes	8.3 ± 1.1	9.6 ± 0.7	11.5 ± 1.1
Mature oocytes (%)	74.7 ± 3.7	75.5 ± 2.0	78.2 ± 1.7
No. of fertilized oocytes	4.6 ± 0.9	7.3 ± 0.8[a]	9 ± 0.9[b]
No. of cleaved oocytes	4.2 ± 0.8	7.1 ± 0.7[a]	8.6 ± 0.9[b]
Fertilization rate (%)	54.4 ± 8.3	74.7 ± 4.5[a]	80.7 ± 3[a]
Cleavage rate (%)	64.7 ± 9.2	89.4 ± 3.8	94.5 ± 1.2[a]
Cycles with no fertilization, n (%)	8 (32)	4 (8)*	0[†]
Transferred embryo rate (%)	42.7 ± 7.4	55.5 ± 4.9[a]	61.0 ± 4
No. of transferred embryo	2.5 ± 0.4	3.3 ± 0.2[a]	4.2 ± 0.1[a]
Transferred embryo's grade	1.7 ± 0.3	1.9 ± 0.1	2.0 ± 0.1
Pregnancy rates (%)	0	0	100

Values are mean ± SEM unless otherwise noted.

Note: Transferred Embryo Rate = transferred embryos/total embryos.

[a] $P<.05$; HS <10% vs. HS >10%NP or HS >10%P. No differences between HS >10%NP and HS >10%P.

[b] $P<.001$; HS <10% vs. HS >10%NP or HS >10%P. No differences between HS >10%NP and HS >10%P.

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Analysis of the IVF Cycles

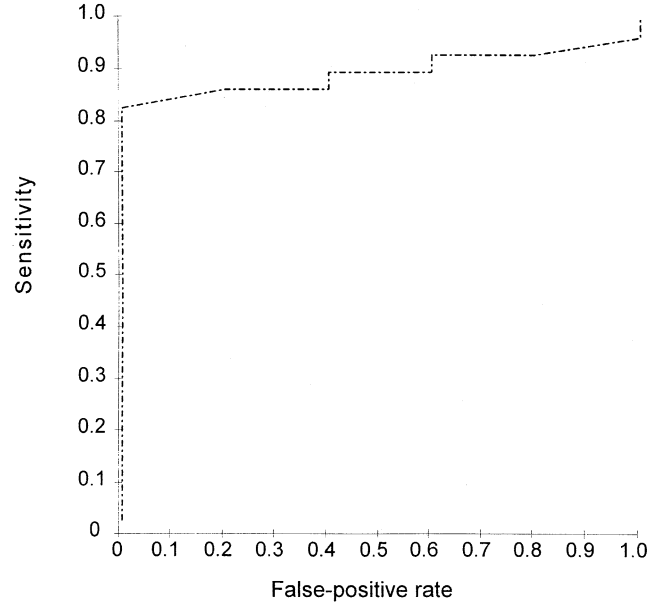
The lack of differences in ovarian factors among the three groups is indicated by the similarities in the number of oocytes retrieved and by the maturational level of the retrieved oocytes (Table 4). The number of fertilized oocytes and the fertilization rates were lower in the HS <10% group compared with in the two control groups, whereas there were no differences between the HS >10%NP and HS >10%P groups. An important difference was apparent in the incidence of couples with no oocyte fertilization, which was 32%, 8%, and 0% in the three groups, respectively ($P=.016$ for the HS <10% vs. HS >10%NP group and $P<.001$ vs. the HS >10%P group). Further, an early attrition of zygote development, most likely related to sperm factors, was detected by considering the cleavage rates, which were 37% and 46% lower in the HS <10% vs. the HS >10%NP and the HS >10%P groups ($P=.03$ and $P<.001$, respectively, Table 4). There was also a difference in the number of cleaved oocytes transferred in the HS <10% compared with the two control groups (42.7% vs. 55.5% and 61%, $P<.001$ vs. the HS <10% group, but no difference between the HS >10%NP and HS >10%P groups). These differences were independent from the grades of the surviving embryos, which were not different among the three groups (Table 4).

ROC Analysis

We further evaluated the predictive value of sperm HspA2 ratio by constructing a ROC curve based on the HspA2 ratios of the HS <10% group and those men in the HS >10%NP and HS >10%P groups whose HspA2 ratios were in the 10%–17%, transitional range (Fig. 2). In line with the 10% HspA2 cutoff value that we arrived at by considering the correlation between sperm CK activity and HspA2 ratios, the ROC cutoff value for failure to cause pregnancy was at the HspA2 ratio of 10.8%, with a specificity of 100%, a sensitivity of 83%, and a positive predictive value of 100%.

FIGURE 2

ROC curve. Area under the curve = 0.9000, standard error = 0.0630.



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DISCUSSION

We have reexamined the value of <10% sperm HspA2 ratios in predicting failure to cause pregnancy in 119 IVF cycles, an association we initially established in 1992 (6). This was of particular interest because in the last decade, a trend has developed in which men with severely oligozoospermic or asthenozoospermic samples have been triaged for ICSI, and couples with moderately diminished sperm concentration and motility are treated with IVF. This has occurred despite the fact that it has become increasingly apparent that adequate motile sperm concentrations alone are not proof of male fertility.

In the HS <10% group in which no pregnancies occurred, the fertilization rates, the cleavage rate of two-pronuclei zygotes, and the proportion of transferable embryos were significantly lower compared with the case of two control groups with male partners who had >10% sperm HspA2 ratios (HS >10%NP and HS >10%P). In line with the diminished sperm maturity in the HS <10% group, a third of the couples had no oocyte fertilization. In contrast, the two control groups of 94 couples with male partners in the mature >10% HspA2 ratio range showed a 46% pregnancy rate. Within the control groups, there were no differences either in the male or female factors or in the IVF data; diminished sperm maturity, determined by the HspA2 ratio, was the only identifiable element predicting pregnancy outcome. Above the apparently critical threshold of 10% HspA2 expression, pregnancies occurred in both the present and in the 1992 study (6). On the basis of the combined data from the two HspA2 studies, we can conclude that in couples in which the man has <10% sperm HspA2 ratio, conventional IVF should be avoided because it can be predicted to fail. Whether the men are normozoospermic or oligozoospermic, ICSI is preferable even if the occurrence of pregnancy is of low likelihood because of diminished sperm maturity. Because of the complexity of sperm functions, it is unclear at the present time whether there are sperm that are deficient in zona pellucida interaction but are less impaired in other fertilizing and embryo support contributions.

Among the 115 male partners of the 119 IVF cycles (4 repeat cycles), only 15 (13%) were oligozoospermic, which appears to be a low number compared with our 1992 study, in which 22 of 84 (26%) men were oligozoospermic. This shift is due to the fact that men with abnormal semen parameters are increasingly being treated with ICSI and hence were not included in this study. The result of this shift was a higher proportion of normozoospermic men with <10% HspA2 ratios and diminished fertility. The proportion of these men increased from 9 of 22 (42%) in 1992 to 13 of 25 (54%) in the HS <10% group of this study. Thus, we have further confirmed the concept that there is infertility in normospermic men that can be detected with objective sperm biochemical markers but not by conventional semen parameters. We can conclude that sperm HspA2 ratio determination is a valuable addition to the workup of a couple, particularly if any type of assisted reproduction procedure, even intrauterine insemination, is considered. The validity of the HspA2 ratio is further strengthened by the fact that both the 1992 and the present HspA2 studies were conducted blindly.

Both CK activity and HspA2 ratio are biochemical markers of spermiogenetic maturation, reflecting the events of male germ cell development that occur in the adluminal compartment of the testis (5). The CK activity is a measure of cytoplasmic retention that is a consequence of the incomplete extrusion of the cytoplasm and diminished sperm cellular maturity (2, 3). The sperm HspA2 ratio reflects the proportion of sperm that have undergone the spermiogenetic changes that occur simultaneously with cytoplasmic extrusion, as indicated by the close correlation between CK activity and HspA2 ratio in this study ($r = -0.82$) and in three previous studies (4, 6, 7). The value of <10% HspA2 is now independently validated by the ROC cutoff value of 10.84% with high specificity, sensitivity, and positive predictive value for failure of IVF pregnancy (100%, 83%, and 100%, respectively).

In the past decade, we and other laboratories have developed a very solid scientific basis for understanding the central role of the developmentally regulated HspA2 chaperone that participates in the function of the synaptonemal complex and the delivery of DNA repair and other enzymes during meiosis and sperm development (5, 8, 14). Also, we have shown that there is a late surge of HspA2 synthesis during terminal spermiogenesis that potentially facilitates protein movements of cytoplasmic extrusion and plasma membrane remodeling in the elongating spermatids (5). Studies directed to the regulation of HspA2 synthesis in men will likely provide a better understanding for new approaches in the diagnosis of male infertility and perhaps male contraception as well.

Immature human sperm show diminished plasma membrane remodeling and zona-binding ability (15, 16), increased rate of aneuploidies (17), an increased rate of lipid peroxidation (10, 18), and consequential DNA fragmentation (8, 20); these collectively cause reduced fertilization rates and adversely affect the paternal contributions to the zygote. Another likely consequence of the low levels of HspA2 in immature sperm is the interrupted delivery of DNA repair enzymes and other proteins that are normally "chaperoned" by the HspA2. Thus, DNA damage also may be linked to abnormal chromatin packaging and histone–protamine exchange in immature or improperly developed spermatozoa. All these factors are known to substantially affect embryo development and maintenance of pregnancies (19–22).

DNA fragmentation and strand breaks may also be detectable with the sperm chromatin structure assay (SCSA), a measure of susceptibility of sperm DNA to low pH–induced denaturation. Indeed, SCSA using computer-interfaced flow

cytometry of acridine orange–labeled sperm cells has also predicted men who have failed to cause pregnancies or whose sperm development has been potentially affected by environmental exposure factors (23, 24). Similar to the sperm CK activity and HspA2 ratio parameters, SCSA has also been useful for the detection of unexplained male infertility; abnormal SCSA parameters predicting diminished fertility were largely independent from the conventional semen analysis parameters (25).

In considering the cellular markers of sperm function, we also examined the relationship between sperm shape as determined by objective morphometry and sperm maturity. Initially, we found that sperm with high CK content (cytoplasmic retention) are also rounder, have larger and asymmetrical heads, and show a higher incidence of amorphous sperm forms (2). Some of these features are also components of the strict morphology evaluation of Kruger (26). More recently, we applied objective, computer-assisted morphometry for the study of sperm attributes that are characteristic of mature and immature sperm as observed via their differences in specific gravity, CK activity, and HspA2 ratio (13).

We hypothesized that there is a relationship between the biochemical markers of sperm maturity and sperm shape based on the completion of various spermiogenetic events. Indeed, in immature sperm in which cytoplasmic extrusion, HspA2 expression, and tail sprouting are all incomplete, the retained extra cytoplasm in the midpiece, plus the shorter tail length, contributed to development-related morphological variations that were well distinguishable by morphometry (13). These relationships among the three objective measures mutually confirm the validity of each of the parameters. However, because morphometry involves labor-intensive preparation of sperm and evaluation of individual sperm by a complex and expensive instrument, and because CK activity measurements (expressed as CK units per million sperm) requires an accurate estimation of sperm density, which is difficult in oligospermic samples, the HspA2 ratio measurements are the easiest and most reliable approach for evaluation of sperm maturity. Indeed, an immunoassay for sperm HspA2 will be available soon.

In summary, we have reexamined the predictive value of sperm HspA2 in IVF pregnancy success. Men in the diminished maturity, <10% HspA2 range failed to cause pregnancies. This confirms the findings of our previous blinded study in 1992. We have also studied a control group of 94 couples in whom the male partners were in the >10% sperm HspA2 ratio (mature) range to assess the potential predictive value of other factors. In this group, there was a 46% pregnancy rate, but there were no male-, female-, or IVF-related parameters that would have predicted pregnancy success or failure. The data suggest that in the treatment of couples with a <10% sperm HspA2 ratio, whether the men are oligozoospermic or normozoospermic, ICSI is the preferred modality, and conventional IVF, which would predictably fail, should be bypassed. The likelihood of achieving pregnancy for men in the <10% HspA2 group, via ICSI, is likely to be lower than in other indications, but the outcome may depend on man-to-man differences in the degree of sperm immaturity; some sperm may be further developed, as sperm populations are known to be heterogeneous in cellular properties. We will study this. It is conceivable that future selection methods aimed at mature sperm will facilitate improved ICSI results in men with diminished-maturity sperm samples.

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